

The Presence of Leukocytes in *Ex Vivo* Assays Significantly Increases the 50-Percent Inhibitory Concentrations of Artesunate and Chloroquine against *Plasmodium vivax* and *Plasmodium falciparum*[▽]

S. Kaewpongsri,^{1†} K. Sriprawat,^{1†} R. Suwanarusk,² D. E. Kyle,³ U. Lek-Uthai,⁴ M. Leimanis,¹
K. M. Lwin,¹ A. P. Phyto,¹ J. Zwang,¹ B. Russell,^{2*} F. Nosten,^{1,5,6} and L. Renia²

Shoklo Malaria Research Unit, Mae Sod, Tak, Thailand¹; Laboratory of Malaria Immunobiology, Singapore Immunology Network (SIgN), A*STAR, Biopolis, Singapore²; Department of Global Health, College of Public Health, University of South Florida³; Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University, Bangkok, Thailand⁴; Centre for Clinical Vaccinology & Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, United Kingdom⁵; and Faculty of Tropical Medicine, Mahidol University, Rajvithi Road, Bangkok, Thailand⁶

Received 10 August 2010/Returned for modification 17 November 2010/Accepted 21 December 2010

***Plasmodium* species *ex vivo* sensitivity assay protocols differ in the requirement for leukocyte removal before culturing. This study shows that the presence of leukocytes significantly increases the 50% inhibitory concentration (IC₅₀) of *P. vivax* and *P. falciparum* to artesunate and chloroquine relative to results with the paired leukocyte-free treatment. Although leukocyte removal is not an essential requirement for the conduct of *ex vivo* assays, its use has important implications for the interpretation of temporal and spatial antimalarial sensitivity data.**

Plasmodium falciparum and *Plasmodium vivax* *ex vivo* sensitivity assays are an important adjunct to *in vivo* antimalarial resistance detection. *Ex vivo* antimalarial sensitivity testing (a specialized form of *in vitro* sensitivity testing) involves the phenotypic comparison of freshly isolated *Plasmodium* spp. cultured to a specified endpoint in the presence and absence of antimalarials. *Ex vivo* assays provide information on the intrinsic sensitivity of malaria parasites, free from confounding variations in patient immune status. Furthermore, in the case of *P. vivax*, *ex vivo* assays are also free from the problematic differentiation between relapse, reinfection, or recrudescence which is needed for *in vivo* testing.

Since the development of *ex vivo* sensitivity antimalarial assays in the 1960s (20), there have been various modifications to the basic method, including changes in the blood medium mixture (BMM) composition (variations in hematocrit, culture medium used, and percent serum added), BMM volume (i.e., 50 μ l versus 200 μ l), and even incubation gas environment (candle jar versus commercial gas). Another important modification to *ex vivo* sensitivity antimalarial assays is the depletion of leukocytes from the patient blood sample prior to addition to the culture medium (26). It was supposed that the depletion of patient leukocytes and platelets removes the possibility that variations in host immune status (i.e., variations in leukocyte count) will confound the result of the sensitivity assay. Addi-

tionally, the removal of leukocytes aids in the microscopic examination of thick films which are used to assess the antimalarial effect on parasite development (26). Furthermore, thick and thin films made from filtered isolates produce “noise-free” images better suited for digital analysis. Despite the growing trend toward complete leukocyte depletion prior to culture (1, 8, 11, 13, 14, 17, 21–23, 25, 27), some groups still utilize patient samples with no or only partial depletion of leukocytes (removal of the buffy coat only) (5, 6, 15, 16, 28). Despite this major dichotomy in *ex vivo* assay methodology, it is not known if the removal of leukocytes influences the antimalarial sensitivity of the parasite or the *ex vivo* growth of *P. vivax* or *P. falciparum*. Consequently, the main objective of this study was to investigate whether the presence of leukocytes affects the IC₅₀ (50% inhibitory concentration) of chloroquine and artesunate against *P. vivax* and *P. falciparum*. It was also important to determine if chloroquine and artesunate significantly affected leukocyte phagocytosis (parasite removal), which, if it occurred in a dose-dependent manner, would confound the assay test results. For example, if higher concentrations of antimalarial inhibited phagocytosis, the parasitemia at lower concentrations would be reduced relative to that with the higher-concentration treatments, counter to the presumed effect of the antimalarial.

This study focuses on a modified version of the original WHO schizont maturation microtest (19), as it remains the most reliable and commonly used methodology for the side-by-side antimalarial sensitivity testing of *P. vivax* and *P. falciparum* in field locations (11, 13, 14, 17, 21, 24). Consequently, the methodology used for this study is based on that of Russell et al. (22), the only major change being the use of a gas mix (N₂, 90%; O₂, 5%; and CO₂, 5%) rather than a candle jar.

* Corresponding author. Mailing address: Laboratory of Malaria Immunobiology, Singapore Immunology Network (SIgN), 8A Biomedical Grove, 03-06 Immunos, Singapore 138648, Singapore. Phone: 65 64070055. Fax: 65 64642056. E-mail: bruce_russell@immunol.a-star.edu.sg.

† These authors contributed equally.

[▽] Published ahead of print on 28 December 2010.

Fifteen *P. vivax* and 10 *P. falciparum* isolates were collected from malaria patients attending the clinics of the Shoklo Malaria Research Unit (SMRU) Mae Sod region of Tak Province in the northwest of Thailand from October 2008 to January 2010. Isolates were collected only from patients with no prior antimalarial therapy and with a microscopically determined parasitemia of $<10,000$ parasites/ μ l. After written consent was obtained, blood samples were collected by venipuncture in 5-ml-volume lithium-heparin tubes and arrived at the culture lab at SMRU within 5 h of collection. An additional 1 ml of blood collected on EDTA anticoagulant was taken from each patient for automated hematology analysis (model pocH-100i; Sysmex Company). Only samples with $>90\%$ of the parasites at the early ring stage (approximately 6 to 8 h postinvasion) were chosen for drug sensitivity testing. Due to this strict criterion, only 8/15 and 8/10 *P. vivax* and *P. falciparum* isolates, respectively, were included in the study. All of the patient samples used in this study had a normal white blood cell count with minimal variation around the median of 5.6×10^3 leukocytes/ μ l (interquartile range [IQR], 4.5 to 6.8 leukocytes/ μ l) observed. Each of the chosen samples was divided into two, and half of the sample, referred to as the "leukocyte removed" treatment, was depleted of platelets and leukocytes by CF11 filtration (26). It should be noted that the CF11 filtration methodology does not alter the stage composition or viability of the isolates (24). The remaining half of each sample was not filtered, and this was referred to as the "leukocytes present" treatment. Parasites in these two treatments were tested in parallel against chloroquine diphosphate (CQ) (molecular weight [MW], 515.9; Sigma-Aldrich) and artesunate (AS) (base MW, 282.3; Holly Pharmaceuticals Co Ltd.), as previously described (22). Artesunate was used in preference to its metabolite, dihydroartemisinin (DHA), as AS is more commonly used in *ex vivo* studies and is significantly more stable on predosed dried drug plates (9). All of the microscopy work was carried out by a skilled microscopist, and quality assurance was done by an expert microscopist (with 15 years of experience in field microscopy). It was not possible to blind the microscopic reads, since the thick films of the "leukocytes present" treatment are very distinct (26).

The clinical samples examined in this study were collected under the ethical guidelines in the approved protocol OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, United Kingdom).

Dose-response curves and IC_{50} s were calculated by fitting the data to a sigmoidal inhibitory E_{max} (maximum effect) pharmacodynamic model using the WinNonlin v. 4.1 (Pharsight Corporation, CA) software program using duplicate well data for each drug concentration. The median IC_{50} s presented in Fig. 1A and 1B and 2B were compared using a Wilcoxon paired-rank test. The median IC_{50} s presented in Fig. 2C and D were compared using a Kruskal-Wallis test and Dunn's *post hoc* analysis. Statistical analysis was carried out and graphics produced using the GraphPad Prism v. 5 software program.

In all cases tested, the presence of leukocytes *ex vivo* increased the antimalarial IC_{50} relative to that for the paired leukocyte-free treatment (Fig. 1). We suggest that the increase in IC_{50} s is simply due to leukocytes reducing the total concentration of antimalarial agent free to act on the parasitized red blood cells (i.e., the leukocytes act as a "biological sink" or

"bio-sponge" for the antimalarials). Chloroquine is preferentially accumulated into leukocytes, such as neutrophils (which make up the majority of leukocytes present in our isolates) and monocytes (7, 18). However, it is not known if artesunate is preferentially accumulated into or neutralized by leukocytes. Interestingly, an earlier *in vitro* study has also shown how the presence of certain cell types with a preferential uptake of artemisinin competes with parasitized erythrocytes for the accumulation of antimalarials (10). In this case study, the presence of α -thalassemic erythrocytes, which have a higher accumulation capacity than infected wild-type red cells, resulted in lower artemisinin concentrations available to kill the parasites and a subsequent increase in artemisinin IC_{50} s. Although we suspect that leukocytes are also reducing the availability of artesunate in our study, we cannot discount the alternative possibility, that leukocytes directly increase the actual susceptibility of the parasite to the antimalarials added.

It is notable that although the chloroquine IC_{50} of *P. falciparum* was increased in the "leukocyte present" treatment, this change did not reach statistical significance. This is probably due to the fact that chloroquine has little effect on these highly resistant *P. falciparum* strains. *Plasmodium falciparum* in this region of Thailand is well known to be highly resistant to chloroquine, where a 300 nM concentration of this antimalarial is not sufficient to inhibit schizont maturation. Therefore, it is less likely that we would be able to detect much of an increase in IC_{50} s over those observed in this study (Fig. 1). In future studies, it would be useful to repeat this experiment with chloroquine-sensitive isolates of *P. falciparum*, which are extremely rare in the study area.

To examine the effect of antimalarials on leukocyte phagocytosis over 42 h of culture ("leukocytes present" treatments only), a randomly selected sample of 100 leukocytes (consisting of neutrophils, lymphocytes, monocytes, eosinophils, and basophils) in the thin films of each species and drug concentration was examined by light microscopy under 100 \times oil immersion. The phagocytic index was defined as the percentage of leukocytes containing at least one parasite or obvious granule of hemozoin normalized to that of the drug-free control (Fig. 2). The parasitemia of each drug-free control was determined before and after culture for 42 h.

Prior to the start of our study, only 0 to 1.3% (range) of the leukocytes contained any detectable hemozoin or parasites. However, after 42 h of drug-free culture, a median (range) of 38.2% (13.9 to 71.0%) or 38.9% (3.8 to 93.1%) of leukocytes contained *P. vivax* or *P. falciparum*, respectively. This resulted in a significant drop in parasitemias of both species over the 42-h incubation (Fig. 2). It is notable that in addition to free merozoites, entire infected red blood cells (IRBCs) were found in the neutrophils and monocytes postculture (Fig. 2). These findings contradict early reports suggesting that only merozoites are readily phagocytosed by granulocytes and that infected red blood cells are relatively protected from phagocytosis (except if damaged) (2). Although we did not observe IRBCs or merozoites in lymphocytes, it was relatively common to find significant quantities of hemozoin phagocytosed by this cell type. There was no indication that the development of ring stages to healthy schizonts was impeded by the presence of leukocytes; there was only a reduction in overall parasitemia. These results support findings of earlier studies showing no

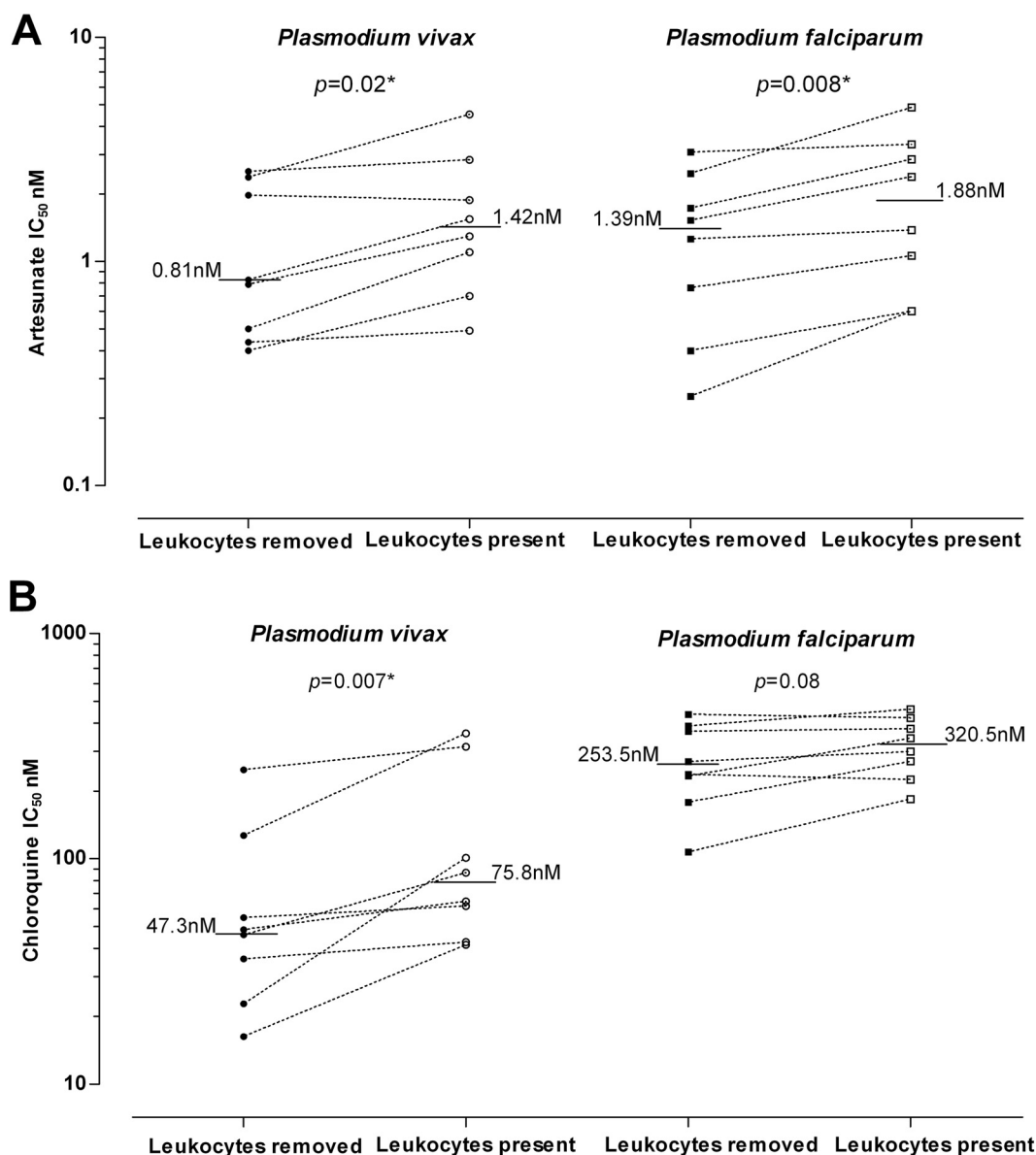


FIG. 1. The effect of leukocyte depletion on the *ex vivo* sensitivity (solid horizontal lines show median IC₅₀ [nM]) of paired isolates of *Plasmodium vivax* ($n = 8$) and *Plasmodium falciparum* ($n = 8$) to artesunate (A) or chloroquine (B). Solid symbols indicate the absence of leukocytes.

marked inhibition of schizont maturation with peripheral blood mononuclear cells or hyperimmune serum (3, 4). Importantly, the concentrations of chloroquine and artesunate used did not significantly affect the phagocytic index (Fig. 2). Although some studies have found that chloroquine and artesunate significantly reduced the phagocytic function of granulocytes, it should be noted that these effects were noted only at concentrations of drug above 100,000 nM chloroquine (12) and 5,000 nM artesunate (29), both of which are well in excess of concentrations used in our study and *in vivo* therapeutic ranges for the treatment of malaria.

The results of our study have important implications for *ex vivo* sensitivity assays. First, the presence of leukocytes can, at least for chloroquine, significantly confound the sen-

sitivity profile of *P. vivax* and *P. falciparum* in *ex vivo* anti-malarial assays by reducing the amount of antimalarial free to act on the parasites. If leukocytes are not removed, sample IC₅₀s may be higher than normally expected, especially in patient samples with particularly high leukocyte counts. Furthermore, the presence of leukocytes prior to sensitivity testing will result in significantly lower parasitemias postculture, making it more difficult to observe the antimalarial effect; this is of particular concern with *P. vivax* isolates, where parasitemias may be close to the microscopic threshold of detection (10 to 50 parasites/ μ l).

The intention of this article is not to advocate the sole use of protocols using leukocyte depletion for *ex vivo* studies on *P. vivax* and *P. falciparum*. In fact, it would be advisable to retain

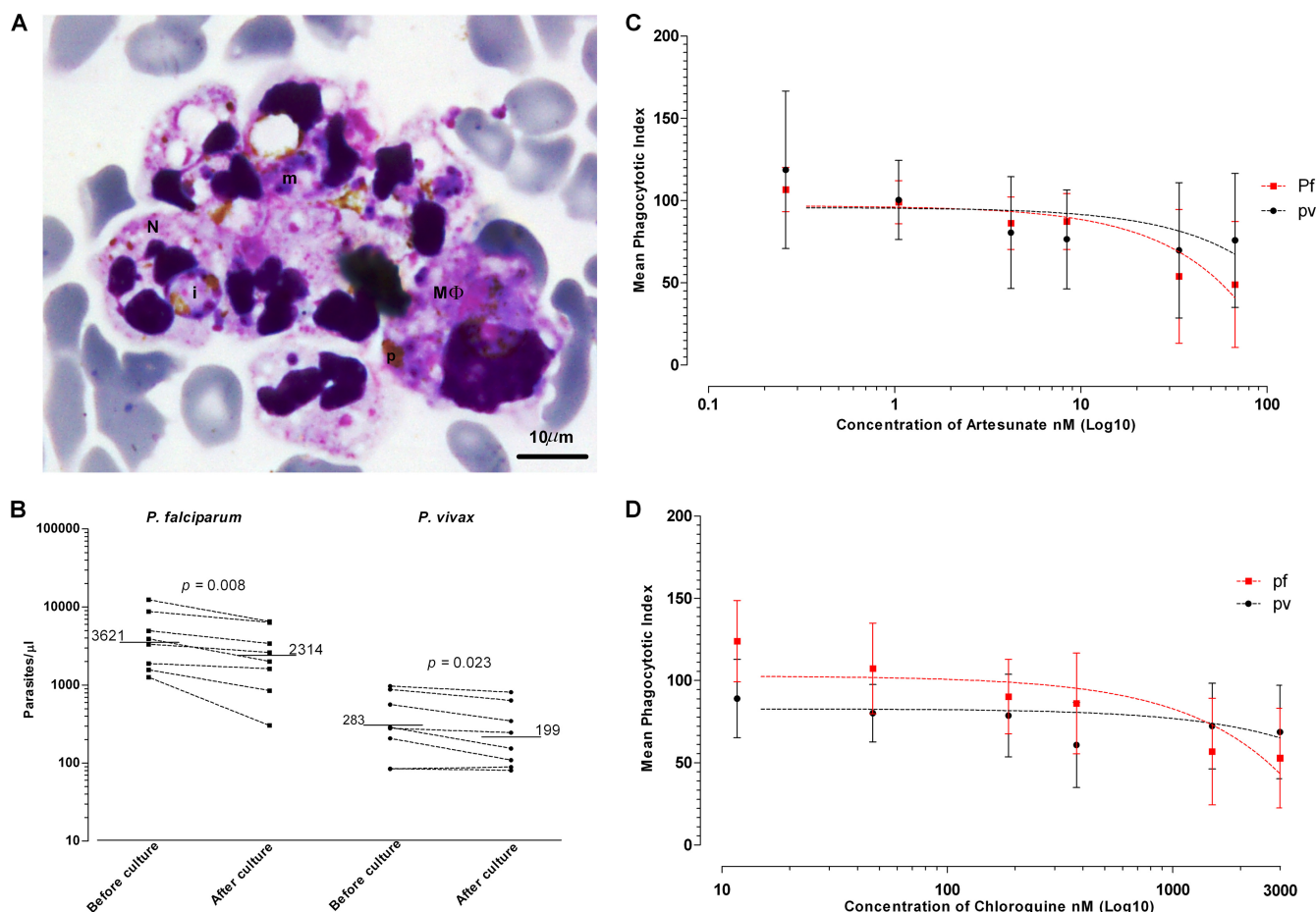


FIG. 2. Neutrophil and monocyte phagocytosis of *Plasmodium vivax* (A) and *Plasmodium falciparum* significantly reduce the parasitemia in *ex vivo* cultures over a period of 42 h (B). The presence of artesunate (C) or chloroquine (D) does not significantly affect the ability of leukocytes to phagocytose infected red blood cells or hemozoin relative to results with the drug-free control (phagocytic index). In the image of a Giemsa-stained thin film (A), examples of a neutrophil (N), monocyte (MΦ), phagocytosed infected red blood cell (i), and hemozoin clump (p) are marked on the photomicrograph.

host leukocytes for studies trying to correlate *ex vivo* sensitivity with clinical therapeutic response. However, we do wish to draw attention to a major dichotomy in *ex vivo* sensitivity protocols that if not taken into account may cause confusion when trying to compare spatial and temporal trends in *Plasmodium* sp. sensitivity profiles. The results of this study are particularly relevant, since there is a trend to move away from the microtest to more automated methods which utilize a differential increase in DNA (stained using dyes such as SYBR green or ethidium bromide) as a measure for parasite growth or inhibition; we predict that the use of leukocyte-depleted isolates (in an effort to remove background signal) will become more frequent.

We thank all of the patients and staff of SMRU for their contribution to this study. We also thank Sue Lee, Nicholas J. White, Arjen Dondorp, and Niklas Lindegaard for helpful discussions.

SIgN is sponsored by the Singapore Agency of Science Technology and Research (A*STAR). SMRU is sponsored by The Wellcome Trust of Great Britain as part of the Oxford Tropical Medicine Research Programme of Wellcome Trust-Mahidol University.

REFERENCES

1. Auliff, A., et al. 2006. Amino acid mutations in *Plasmodium vivax* DHFR and DHPS from several geographical regions and susceptibility to antifolate drugs. *Am. J. Trop. Med. Hyg.* **75**:617–621.
2. Bass, C. C., and F. M. Johns. 1912. The cultivation of malarial plasmodia (*Plasmodium vivax* and *Plasmodium falciparum*) in Vitro. *J. Exp. Med.* **16**: 567–579.
3. Binh, V. Q., A. J. Luty, and P. G. Kremsner. 1997. Differential effects of human serum and cells on the growth of *Plasmodium falciparum* adapted to serum-free in vitro culture conditions. *Am. J. Trop. Med. Hyg.* **57**:594–600.
4. Capps, T. C., and J. B. Jensen. 1983. Storage requirements for erythrocytes used to culture *Plasmodium falciparum*. *J. Parasitol.* **69**:158–162.
5. Chotivanich, K., et al. 2004. In vitro efficacy of antimalarial drugs against *Plasmodium vivax* on the western border of Thailand. *Am. J. Trop. Med. Hyg.* **70**:395–397.
6. Druilhe, P., P. Brasseur, C. Blanc, and M. Makler. 2007. Improved assessment of *Plasmodium vivax* response to antimalarial drugs by a colorimetric double-site plasmodium lactate dehydrogenase antigen capture enzyme-linked immunosorbent assay. *Antimicrob. Agents Chemother.* **51**:2112–2116.
7. French, J. K., N. P. Hurst, M. L. O'Donnell, and W. H. Betts. 1987. Uptake of chloroquine and hydroxychloroquine by human blood leukocytes in vitro: relation to cellular concentrations during antirheumatic therapy. *Ann. Rheum. Dis.* **46**:42–45.
8. Hasugian, A. R., et al. 2009. In vivo and in vitro efficacy of amodiaquine monotherapy for treatment of infection by chloroquine-resistant *Plasmodium vivax*. *Antimicrob. Agents Chemother.* **53**:1094–1099.
9. Houze, S., et al. 2007. Shelf life of predosed plates containing mefloquine,

- artemisinin, dihydroartemisinin, and artesunate as used for in vitro *Plasmodium falciparum* susceptibility assessment. *J. Clin. Microbiol.* **45**:2734–2736.
10. Kamchonwongpaisan, S., G. Chandra-ngam, M. A. Avery, and Y. Yuthavong. 1994. Resistance to artemisinin of malaria parasites (*Plasmodium falciparum*) infecting alpha-thalassemic erythrocytes in vitro. Competition in drug accumulation with uninfected erythrocytes. *J. Clin. Invest.* **93**:467–473.
 11. Kosaisavee, V., et al. 2006. *Plasmodium vivax*: isotopic, PicoGreen, and microscopic assays for measuring chloroquine sensitivity in fresh and cryo-preserved isolates. *Exp. Parasitol.* **114**:34–39.
 12. Labro, M. T., and C. Babin-Chevaye. 1988. Effects of amodiaquine, chloroquine, and mefloquine on human polymorphonuclear neutrophil function in vitro. *Antimicrob. Agents Chemother.* **32**:1124–1130.
 13. Leimanis, M. L., et al. 2010. *Plasmodium vivax* susceptibility to ferroquine. *Antimicrob. Agents Chemother.* **54**:2228–2230.
 14. Lek-Uthai, U., et al. 2008. Stronger activity of human immunodeficiency virus type 1 protease inhibitors against clinical isolates of *Plasmodium vivax* than against those of *P. falciparum*. *Antimicrob. Agents Chemother.* **52**:2435–2441.
 15. Noedl, H., et al. 2001. In vivo-in vitro model for the assessment of clinically relevant antimalarial cross-resistance. *Am. J. Trop. Med. Hyg.* **65**:696–699.
 16. Noedl, H., et al. 2006. Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. *Am. J. Trop. Med. Hyg.* **75**:1205–1208.
 17. Price, R. N., et al. 2010. In vitro activity of pyronaridine against multidrug-resistant *Plasmodium falciparum* and *Plasmodium vivax*. *Antimicrob. Agents Chemother.* **54**:5146–5150.
 18. Raghoebar, M., W. B. van den Berg, and C. A. van Ginneken. 1987. Alteration of chloroquine accumulation in human polymorphonuclear leucocytes under inflammatory conditions. *Int. J. Tissue React.* **9**:255–261.
 19. Rieckmann, K. H., G. H. Campbell, L. J. Sax, and J. E. Mrema. 1978. Drug sensitivity of *Plasmodium falciparum*. An in-vitro microtechnique. *Lancet* **i**:22–23.
 20. Rieckmann, K. H., et al. 1968. Effects of chloroquine, quinine, and cycloguanil upon the maturation of asexual erythrocytic forms of two strains of *Plasmodium falciparum* in vitro. *Am. J. Trop. Med. Hyg.* **17**:661–671.
 21. Rottmann, M., et al. 2010. Spiroindolones, a potent compound class for the treatment of malaria. *Science* **329**:1175–1180.
 22. Russell, B., et al. 2008. Determinants of in vitro drug susceptibility testing of *Plasmodium vivax*. *Antimicrob. Agents Chemother.* **52**:1040–1045.
 23. Russell, B. M., et al. 2003. Simple in vitro assay for determining the sensitivity of *Plasmodium vivax* isolates from fresh human blood to antimalarials in areas where *P. vivax* is endemic. *Antimicrob. Agents Chemother.* **47**:170–173.
 24. Sharrock, W. W., et al. 2008. *Plasmodium vivax* trophozoites insensitive to chloroquine. *Malar. J.* **7**:94.
 25. Siswantoro, H., et al. 2011. In vivo and in vitro efficacy of chloroquine for *Plasmodium malariae* and *P. ovale* in Papua, Indonesia. *Antimicrob. Agents Chemother.* **55**:197–202.
 26. Sriprawat, K., et al. 2009. Effective and cheap removal of leukocytes and platelets from *Plasmodium vivax* infected blood. *Malar. J.* **8**:115.
 27. Suwanarusk, R., et al. 2007. Chloroquine resistant *Plasmodium vivax*: in vitro characterisation and association with molecular polymorphisms. *PLoS One* **2**:e1089.
 28. Tسانور, O., et al. 2002. An in vitro system for assessing the sensitivity of *Plasmodium vivax* to chloroquine. *Acta Trop.* **83**:49–61.
 29. Wenisch, C., B. Parschalk, K. Zedwitz-Liebenstein, W. Wernsdorfer, and W. Graninger. 1997. The effect of artemisinin on granulocyte function assessed by flow cytometry. *J. Antimicrob. Chemother.* **39**:99–101.